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Examining population genetic structure of the pitcher plant mosquito (Wyeomyia smithii) across multiple spatial scales using AFLP markers

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

EXAMINING POPULATION GENETIC STRUCTURE OF THE PITCHER PLANT MOSQUITO (*WYEOMYIA SMITHII*) ACROSS MULTIPLE SPATIAL SCALES USING AFLP MARKERS

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by

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Abstract

The population genetic structure of the pitcher plant mosquito (*Wyeomyia smithii* Coquillett 1901) provides insight into its dispersal patterns and behaviour. I developed a suite of 117 amplified fragment length polymorphism (AFLP) markers for *W. smithii* and, using larvae collected from purple pitcher plants in Algonquin Provincial Park (Ontario, Canada), I studied the population genetic structure of the mosquito across multiple spatial scales. At the finest scale I examined genetic differentiation among samples from different leaves within a single plant, and at the largest scale I examined differentiation among samples from groups of peatlands ~ 26 km apart. Samples from different peatlands, even distant ones, displayed low genetic differentiation, suggesting the mosquito disperses widely among peatlands in a landscape. Significant genetic differentiation among leaves within plants was associated with high relatedness of larvae occurring in the same leaf, suggesting that females lay their eggs in clumps.

Keywords

AFLP, genetic structure, pitcher plant mosquito, *Sarracenia purpurea*, spatial scales, *Wyeomyia smithii*

Co-Authorship

I completed this thesis under the supervision of Dr. Nusha Keyghobadi who will be a co-author on manuscripts arising from the thesis. Dr. Keyghobadi provided advice and feedback on all aspects of my study. Dr. Gordana Rasic will be another co-author of any manuscripts arising from my thesis, as I used *Wyeomyia smithii* larvae she collected in August 2009, and I also made use of pitcher plant density data that she collected. I sorted the *W. smithii* larvae from midge larvae and performed all DNA extractions. I developed a novel suite of amplified fragment length polymorphism (AFLP) markers for *W. smithii* and used them to genotype the larvae. I also planned and completed, with guidance from Dr. Keyghobadi, all aspects of data analysis, including the scoring and determination of error rate for AFLPs, estimation of population genetic structure, and other statistical analyses.

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List of Abbreviations

AFLP	amplified fragment length polymorphisms
AIC_c	Akaike Information Criterion
AMOVA	analysis of molecular variance
BAB	Bab Lake
bp	base pair
BUG	Buggy
DL	Dizzy Lake
F_{ST}	fixation index (measure of genetic differentiation)
GLM	generalized linear model
H_j	expected heterozygosity
IBD	isolation-by-distance
MIN	Minor Lake
min	minute
ML	Mizzy Lake
PCR	polymerase chain reaction
PPL	proportion of polymorphic loci
RADseq	restriction site-associated DNA tag technology
RAD tag	restriction site-associated DNA tag
rfu	relative fluorescence units
RS	Roadside
s	second
SB	Spruce Bog
SNP	single nucleotide polymorphism
SYS1	System 1
SYS2	System 2
WR	West Rose

List of Software Packages

AFLPdat	http://www.nhm.uio.no/english/research/ncb/aflpdat/
AFLPScore	http://www.shef.ac.uk/molecol/software/aflpscore
AFLP-SURV	http://www.ulb.ac.be/sciences/lagev/aflp-surv.html
ArcGIS 10.0	Environmental Systems Resource Institute (Redlands, CA)
GENALEX	http://www.anu.edu.au/BoZo/GenAlEx/
GeneMapper	Applied Biosystems (Forest City, CA)
HIERFSTAT	http://www2.unil.ch/popgen/softwares/hierfstat.htm
R Project	http://www.r-project.org/

Chapter 1: Introduction

1.1 Population genetic structure, gene flow, and dispersal

Population genetic structure refers to the distribution of genetic variation within and among populations of a species, and can provide important insight into the ecology and behaviour of that species. Given the genetic variation introduced by mutation, three main evolutionary processes determine patterns of population genetic structure: selection, genetic drift, and gene flow (Hartl & Clark 2007). Selection acts on available genetic variation, so that alleles favourable for an organism's survival and reproduction are maintained or increased in frequency, while deleterious alleles are eliminated (Hamilton 2009). Similar selection pressures in different populations lead to similar allele frequency distributions at the loci under selection, while different selection pressures in different populations can lead to divergent allele frequencies (Hendry et al. 2007). Genetic drift is a change in allele frequencies between generations as a result of the random sampling of alleles from a finite population (Charlesworth 2009). Genetic drift results in populations losing genetic variation over generations and this loss of diversity is accelerated in small populations (Frankham et al. 2010). Drift acting in independent populations will, on average, make those populations more genetically divergent (Hamilton 2009). Gene flow, or gene migration, is the movement of alleles from one population to another. Gene flow introduces potentially novel genetic variation into populations, and the transfer of alleles can make spatially distinct populations more genetically similar to one another (Slatkin 1985). Overall, the combined effects of selection, drift and gene flow determine how much genetic variation is maintained within and among populations (Hartl & Clark 2007; Hamilton 2009). While drift and gene flow affect all parts of an organism's genome equally, selection will affect each locus differently depending on the variation available at the locus and the particular relationships among alleles of that locus, individual phenotype, and fitness (Bonin et al. 2006).

The evolutionary forces of drift, gene flow, and selection are in turn affected by underlying ecological processes. For example, selection is determined by differential survival and reproduction of individuals under a particular set of environmental conditions. Likewise,

effective population size determines the rate at which genetic variation is eroded by genetic drift: it is the size of an 'ideal' population (i.e., meeting all Hardy-Weinberg assumptions) that would lose genetic diversity at the same rate as that of the observed population (Wright 1931; Vucetich et al. 1997). Effective population size is highly dependent on census population size (the actual number of individuals), as well as population fluctuations (i.e., variation in population size) over time, sex ratio of breeding adults, and mating patterns (Hartl & Clark 2007; Hamilton 2009). Such ecological processes and variables are of significant interest because of their important role in determining population, community and ecosystem patterns. For example, rates of individual survival and reproduction, as well as population fluctuations and mating patterns, ultimately affect the capacity for a population's persistence and growth.

Gene flow is very strongly linked to the ecological process of dispersal, and dispersal in turn is a key factor influencing population and community dynamics. The term 'dispersal' is used in different contexts in the literature. For example, in birds and mammals, 'natal dispersal' refers to movements away from the area of an individual's birth to the area where it first breeds, and 'breeding dispersal' refers to movements between successive breeding areas. Here, I define dispersal more generally as the movement of individuals among habitat patches (Bowler & Benton 2005). Potential factors motivating dispersal include kin competition, inbreeding avoidance, resource competition, and environmental stochasticity (Bowler & Benton 2005). Dispersal, in turn, is a crucial ecological process affecting the growth rate and size of local populations, patch colonisation, synchrony of population size changes, and persistence of regional population networks (Levins 1969; Hanski 1999; Bowler & Benton 2005; Matter & Roland 2010), as well as inter-species interactions (Huffaker 1958; Kareiva 1987). When accompanied by reproduction in the new location, dispersal becomes synonymous with gene flow, which is a key determinant of genetic structure, counteracting the differentiation of populations caused by genetic drift or selection (Stenseth & Lidicker Jr 1992; Schaal et al. 1998; Freeland 2005; Croteau 2010).

Despite its importance, dispersal can be challenging to study directly. Aside from being technically difficult, marking and following organisms, or fitting them with tracking devices, are time-consuming and expensive (Mech 1983). Key drawbacks of marking techniques are the difficulty in relocating marked individuals (Bullock et al. 2002), as well as limitations in

the spatial and temporal scales that can be covered (Bossart & Prowell 1998). These issues are particularly pronounced in small, flying insects. Tracking devices have been designed only for larger insects such as butterflies and bumblebees (Osborne et al. 1999; Wang et al. 2011). Most mark-recapture-release studies also use tags that are too large for use on many of the smaller insects, such as mosquitoes and midges, or the application of tags is onerous and time-consuming (Hagler & Jackson 2001). Even the initial live capture of some very small insects can be challenging.

An alternative and widely used approach to understanding patterns of dispersal in natural populations is to estimate population genetic structure using gene frequency data, and to infer indirectly the extent of gene flow and hence, dispersal (Whitlock & McCauley 1999). High genetic similarity of populations can potentially be attributed to high levels of gene flow and dispersal, while high genetic divergence among populations can be attributed to lower levels of gene flow and dispersal (Keyghobadi et al. 2003). While population genetic differentiation can also be affected by selection and genetic drift, correlations between direct estimates of dispersal and population genetic structure are strong and pervasive. Furthermore, any confounding effect of selection can be reduced by using neutral or genome-wide genetic markers. Meta-analyses have confirmed a significant and consistent correlation between genetic differentiation and dispersal, both within phytophagous insects (Peterson & Denno 1998) as well as across 333 vertebrate and invertebrate animals (Bohonak 1999). As a result, molecular markers have allowed insights into insect dispersal where other means have failed (Bullock et al. 2002). Overall, studies of population genetic structure can provide important insights into key ecological processes, particularly dispersal, and are especially valuable when direct observation or quantification of those processes is difficult. In this thesis, I examine population genetic structure of an insect, the pitcher plant mosquito (*Wyeomyia smithii* Coquillett 1901), whose dispersal is very challenging to study using direct measures.

1.2 The purple pitcher plant and its inhabitants

The carnivorous purple pitcher plant, *Sarracenia purpurea* L. (Sarraceniaceae), is found in wetlands, including low-nutrient peatlands, from northern Canada to the Gulf of Mexico, and from the east of the Rocky Mountains to the Atlantic Coast (Steward & McWade 1960;

Bradshaw & Holzapfel 2008; Hoekman et al. 2009). Each leaf on the plant is modified into the shape of a pitcher that collects rainwater, creating a phytotelma (i.e., a small water body held within a plant structure). The pitcher-shaped leaves of this herbaceous perennial form a rosette that defines an individual plant (Giberson & Hardwick 1999). In early spring, the purple pitcher plant produces new pitchers that begin collecting water once they are open (Fish & Hall 1978). Each pitcher typically survives on the plant for two years (Giberson & Hardwick 1999), so each individual plant represents a mixture of new and old pitchers.

Many invertebrates, such as ants, are attracted to the plant's pitchers by UV reflectance and nectar, but become trapped in the rainwater-filled structures and eventually decompose. Nutrients released by this decomposition process become available to the plant, and carnivory by the plant is thought to be an adaptation to low-nutrient, particularly low-nitrogen, environments (Bradshaw & Creelman 1984; Chapin & Pastor 1995). In contrast, the same leaves provide the exclusive habitat for larvae of several insects, as well as specialized mites, rotifers, and bacteria (Giberson & Hardwick 1999; Hoekman et al. 2009).

Decomposing invertebrate prey form the base of an aquatic food web that becomes established within each leaf of *S. purpurea* (Giberson & Hardwick 1999; Gotelli et al. 2011). The larvae of three highly specialized insects live within the leaves of *S. purpurea* and play key roles in this food web. Even though these pitcher plant inquilines (i.e., organisms living inside another organism without harming the host) are all limited by food supply, they coexist by partitioning their habitat spatially within a pitcher and feeding on material in different stages of decay (Heard 1994a). Larvae of the pitcher plant flesh fly, *Fletcherimyia fletcheri*, and pitcher plant midge, *Metriocnemus knabi*, feed directly on the decaying invertebrate carcasses (Heard 1994a; Gotelli et al. 2011). While *F. fletcheri* larvae scavenge newly captured insects floating on the water's surface, *M. knabi* larvae feed on solid material that has descended to the bottom of the pitcher (Fish & Hall 1978; Bradshaw 1983; Heard 1994a). In contrast, *W. smithii* larvae, as active swimmers, filter-feed on particulate matter, microorganisms, and protozoans in the water column of the pitcher (Heard 1994a; Buckley et al. 2004). The mosquito benefits from the feeding activity of the midges, which increases particulate and microbial matter in the water column. Food supply for the midge, however, is unaffected by the mosquito. Therefore, the relationship between these two insects has been described as a 'processing chain commensalism' (Heard 1994a). Furthermore, while the plant

is not dependent on the inquiline insects for prey digestion (Gallie & Chang 1997), it does benefit from their presence as rates of digestion and nutrient availability increase when the inquilines are present (Adams & Smith 1977; Bradshaw & Creelman 1984). As such, the relationship between the plant and its insect inhabitants is sometimes referred to as mutualistic (Bradshaw & Creelman 1984).

The phytotelmata of the purple pitcher plant can serve as natural microcosms for testing hypotheses regarding community and ecosystem processes (e.g., Kneitel & Miller 2002, 2003), as well as for understanding how fragmented habitats affect dispersal (Srivastava et al. 2004). Microcosms are small, contained habitats that provide high tractability and clearly delineated areas for studying populations and species interactions (Srivastava et al. 2004). A particularly useful characteristic of the purple pitcher plant as a microcosm is that the habitat of the pitchers' inhabitants can be described at multiple, nested scales. In some areas, peatlands tend to be very discrete cover types in the landscape. These, in turn, contain 'clusters' of patchily distributed pitcher plants. Within these clusters, the plants define very discrete patches of habitat and are themselves composed of discrete leaves. The habitat can therefore be described at multiple levels from leaves, to plants, to clusters, to peatlands, and to systems of peatlands. Nested spatial scales and well-defined habitat patches make the pitcher plant system useful in studies of community ecology and landscape ecology, which aim to understand species distribution, species abundance, and community composition (Krawchuk & Taylor 2003; Buckley et al. 2004).

In general, the scale of any ecological or evolutionary study is very important in determining the patterns and processes that can be revealed (Wiens 1989). The extent (the entire area included in a study) and grain (the size of individual units of observation) of a study are the upper and lower limits of resolution, and they jointly determine our ability to detect patterns (Wiens 1989). Since inferences cannot be accurately made beyond the extent or grain of an investigation (Wiens 1989), there is considerable benefit to be gained from study systems that allow simultaneous examination of patterns at multiple spatial scales. The hierarchical spatial structure of the pitcher plant system is a particularly important feature in studying the dispersal of insects associated with the plant, as dispersal occurs over a range of spatial scales and may affect ecological and evolutionary processes differently at different scales (Cadotte & Fukami 2005).

More recently, the insect inhabitants of the purple pitcher plant have been used in the field of landscape genetics, which focuses on how landscape characteristics influence the microevolutionary processes that structure genetic variation across space (Manel et al. 2003; Rasic & Keyghobadi 2012a, b). In particular, the hierarchical spatial arrangement of the insects' habitat provides an excellent system in which to understand how population genetic structure of a species may vary across spatial scales, and in response to changes in habitat structure. Many studies describe population genetic structure at more than one scale, but the majority of these studies incorporate up to only three levels (Rasic & Keyghobadi 2012b). In contrast, the pitcher plant system naturally consists of five, objectively defined scales of habitat (leaf, plant, cluster, peatland, system). Furthermore, comparative studies on *S. purpurea* inquilines can shed light on the role of dispersal in mediating the relationship between habitat structure and population genetic structure, as the dispersal behaviours and abilities of the three pitcher plant insects appear to differ (Krawchuk & Taylor 2003).

1.3 Pitcher plant mosquito

The pitcher plant mosquito, *W. smithii*, is the best-studied member of the purple pitcher plant inquiline community (Harvey & Miller 1996). It is also perhaps the most widely known of the purple pitcher plant inhabitants, being the first species shown to have a genetic change in response to recent, rapid climate change, postponing diapause as growing seasons lengthen (Bradshaw & Holzapfel 2001). Overall, *W. smithii* has been the subject of extensive ecological and evolutionary research, particularly in studies of community structure and the evolution of life history traits such as diapause (e.g., Addicott 1974; Fish & Hall 1978; Bradshaw 1983; Bradshaw & Creelman 1984; Heard 1994a; Heard 1994b; Bradshaw et al. 1998; Buckley et al. 2004; Bradshaw & Holzapfel 2008).

Wyeomyia smithii is found from Florida to Labrador, and west to Manitoba (Giberson & Hardwick 1999). While *W. smithii* is active as a winged adult in the summer, its larvae are found only in water-bearing leaves of pitcher plants, where they complete their pre-adult development (Steward & McWade 1960; Zani et al. 2005; Emerson et al. 2010). In the northern parts of its range, the mosquito is restricted to the purple pitcher plant, *S. purpurea*, which is the only pitcher plant found in those regions. However, in the southern ends of its

distribution *W. smithii* may also be found in other species of pitcher plant (Juniper et al. 1989). Diapausing third- or fourth-instar larvae overwinter in the pitchers, and though they can survive freezing (Steward & McWade 1960), their survival at northern latitudes appears dependent on adequate insulating snow cover (Heard 1994b). Under optimum food conditions, larvae require about 3 weeks to develop at 23˚C, but development slows when food is limited (Wallis & Frempong-Boadu 1967). Development is also temperature-dependent, occurring more rapidly in warmer areas (Kingsolver 1979). In the northern range of the species, the mosquito is univoltine and adult females do not blood-feed, while in southern regions it is multivoltine, and adult females do blood-feed (Giberson & Hardwick 1999).

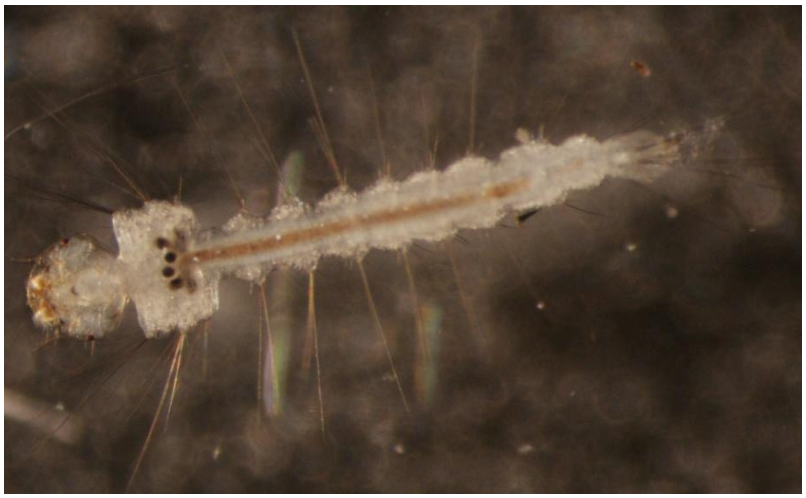

Figure 1.1 *Wyeomyia smithii* larva collected from a pitcher of *Sarracenia purpurea*. Photo credit: Katie Millette.

Female *W. smithii* obligately oviposit into the leaves of the host plant and are thought to lay a single egg, or else a very small clutch of eggs, per leaf (Heard 1994b). Oviposition decisions by *W. smithii* occur at several spatial scales, depending on both meso-scale (e.g. plant density) and fine-scale (e.g. leaf length) factors, with mosquito larval abundance increasing with plant density and leaf length (Trzcinski et al. 2003). Longer leaves signify greater resource availability, as they capture more insects and are less likely to dry out during the summer (Kingsolver 1979; Trzcinski et al. 2003). Females tend to favour younger, larger pitchers for oviposition; interestingly, pitcher age is negatively correlated with pitcher length and hood area (Nastase et al. 1995). At a broader scale, landscape features influence patterns

of *W. smithii* larval distribution; abundance of mosquitoes decreases as peatland size decreases, especially in peatlands surrounded by vegetation less than 2 m tall (Miner & Taylor 2002).

With erratic flight patterns, adult *W. smithii* are hypothesized to be weak fliers that will remain within a single peatland (a scale of hundreds of metres) during their lifetime and that seldom move among peatlands (Bradshaw 1983; Istock & Weisburg 1987; Krawchuck & Taylor 2003; Ragland & Kingsolver 2008). Direct estimates of movement or dispersal in *W. smithii*, such as by mark-recapture, are limited, likely because of the difficulty of handling and tracking adults (due to their small size and sensitivity to handling). In a release-recapture experiment, Krawchuk & Taylor (2003) estimated a mean dispersal distance of only 11 m, and a maximum dispersal distance of 84 m, for *W. smithii*. However, their recapture instances were very low (only 4%), making it difficult to draw firm conclusions about *W. smithii* dispersal, particularly between different peatlands. Hypothesized low dispersal ability in combination with patchily distributed habitat suggest that gene flow in *W. smithii* may also be limited; certainly at a continental scale, the mosquito exists as many strongly isolated populations across its range (Istock & Weisburg 1987; Ragland & Kingsolver 2008).

1.4 Population structure, gene flow, and dispersal in the pitcher plant mosquito

My **objective** was to examine population genetic structure of *W. smithii,* and to make inferences about gene flow and dispersal, across multiple spatial scales using genetic markers called amplified fragment length polymorphisms (AFLPs).

Unlike most populations, which cannot be objectively defined at multiple naturally occurring, distinct spatial scales, *W. smithii* can be sampled at different naturally occurring spatial levels (Buckley et al. 2004), as described previously.

Relatively few studies have focused on understanding patterns of population genetic structure in *W. smithii*, despite the significant insight that an understanding of genetic structure could provide into dispersal and population dynamics. Indeed the spatial genetic structure of the mosquito has been investigated primarily at a phylogeographic scale (Armbruster et al. 1998;

Emerson et al. 2010) and very few studies have examined smaller spatial scales such as within peatlands or among nearby peatlands (Istock & Weisburg 1987). Armbruster et al. (1998) examined allozyme variability of 34 populations of *W. smithii*, distinguishing the Gulf Coast, lowland North Carolina, and northern populations as distinct and separate groupings. They also found that average allozyme heterozygosity was high and similar within populations in the southern region (30-40˚N), but declined north of 40˚N latitude. In order to resolve the phylogeographic history associated with the postglacial range expansion of the mosquito, Emerson et al. (2010) used restriction site-associated DNA tag (RADSeq) technology to isolate single nucleotide polymorphisms (SNPs) throughout *W. smithii*'s genome: they confirmed the subdivision of the mosquito into northern and southern groups, which themselves contain different clades. Finally, Istock & Weisburg (1987) studied twenty-nine *W. smithii* populations and included analyses across scales of the spatial hierarchy of habitats, but using only two enzyme loci. They found structuring of enzyme variation at a continental scale, but little differentiation within peatlands or among peatlands at a regional scale (up to 40 km), patterns which they ascribed to the combined effects of selection and drift.

My work is different from previous population genetic work on *W. smithii*. In contrast to Istock & Weisburg (1987), I used more powerful (genome-wide) molecular markers as well as a fully nested sampling design. Additionally, my work is much smaller-scale (e.g. multiple leaves on the sample plant) than the studies by Armbruster et al. (1998) and Emerson et al. (2010). My contribution is important to providing a fuller understanding of the ecology and evolution of this well-studied insect.

My work also occurs within the context of previous studies of population genetic structure in the pitcher plant midge (*M. knabi*) and flesh fly (*F. fletcheri*), conducted at a similar spatial extent. Rasic & Keyghobadi (2012b) detected patterns of genetic differentiation at both fine and broad scales for *M. knabi* using microsatellite markers: they observed significant differentiation of two peatland systems 26 km apart, as well as differentiation among clusters in a peatland, plants within a cluster, and leaves of a plant. Rasic & Keyghobadi (2012a) also used microsatellites to reveal small but significant differentiation between systems (26 km apart) as well as among peatlands within a system (up to about 7 km apart) for *F. fletcheri*, suggesting limited dispersal among peatlands at such distances. Isolation-by-distance was

highly significant among peatlands at larger spatial scales (15-20 km). Relative to the other two pitcher plant insects, I expected to find intermediate genetic structure for *W. smithii*, which is intermediate in size and predicted dispersal ability between *M. knabi* and *F. fletcheri* (Hamilton & Duffield 2002; Krawchuk & Taylor 2003).

1.5 Hypotheses and predictions

I hypothesized that *W. smithii*, as apparently poor fliers, have weak dispersal abilities. Therefore I predicted that I would observe genetic differentiation of *W. smithii* samples collected from different peatlands and even different clusters of pitcher plants within a peatland. At these scales, I also predicted that mosquitoes would exhibit patterns of isolation-by-distance (IBD), which is defined as the increase in genetic differentiation between individuals as the geographic distance between them increases, and which is a consequence of spatially limited gene flow (Wright 1946).

Furthermore, oviposition decisions of females could affect the dispersion of related larvae, and therefore genetic structure of larval samples, at fine spatial scales (among leaves and plants within clusters). Given the close association of *W. smithii* to pitcher plants, I hypothesized that the availability of host plants would influence female oviposition decisions, and thus genetic structure at smaller spatial scales, as has been shown to be the case for *M. knabi* (Rasic & Keyghobadi 2012b). In *M. knabi*, in instances of low plant density, females appear to deposit most of their eggs into one leaf or plant, perhaps to avoid moving long distances to oviposit (Rasic & Keyghobadi 2012b). I predicted a similar phenomenon in *W. smithii*. Consequently, in peatlands that have lower pitcher density, I expected to observe more highly related larvae within single pitchers, which would result in greater differentiation among larval samples from different pitchers within a single plant.

1.6 Amplified fragment length polymorphisms (AFLPs)

Currently, two genetic marker systems, microsatellites and amplified fragment length polymorphisms (AFLP), are most commonly used to study the genetic structure of, and gene

flow among, natural populations within species. Microsatellites are highly variable, co-dominant markers that have been used extensively over the past 20 years (Selkoe & Toonen 2006). The development of microsatellite markers for *W. smithii*, however, has been unsuccessful (Rasic 2011), and this is hypothesized to be due to repetitive DNA and similarities among microsatellite flanking regions, leading to multiple locus amplifications and unclear banding patterns (Meglecz et al. 2007; Rasic 2011). Therefore, I developed AFLP markers for the analysis of population genetic structure in *W. smithii.*

Zabeau & Vos (1993) originally described the AFLP protocol, which is a powerful DNA fingerprinting technique based on the amplification of genomic restriction fragments through polymerase chain reaction (PCR). The AFLP protocol produces a unique and reproducible DNA profile for each individual (Vos et al. 1995). Allowing efficient and simultaneous analysis of a large number of genetic loci, the AFLP procedure is useful in determining differences among populations, including very closely related ones (Vos et al. 1995). The AFLP technique has several advantages, perhaps the most important being that it can be applied to DNA of any origin, without prior sequence knowledge (Vos et al. 1995). Furthermore, AFLPs are multilocus markers that screen very large numbers of loci in the genome, typically over one hundred (Mueller & Wolfenbarger 1999). A trade-off is that AFLPs are dominant markers, meaning that heterozygotes cannot be distinguished from some homozygotes, therefore complicating some population genetic analyses (Mueller & Wolfenbarger 1999). The targeted regions of the genome are anonymous to the investigator; the AFLP technique simply produces fragments of varying length within the genome (Allan & Max 2010). Nonetheless, the high resolution of the AFLP protocol allows for the identification of even very small genetic differences within a group of organisms: because so many loci are generated, at least some loci will be found in variable regions (Mueller & Wolfenbarger 1999). Previous studies have successfully used AFLPs to elucidate population genetic differentiation in a range of taxa and at various spatial scales. For example, Crawford et al. (2011) employed AFLPs at a fine scale to investigate the genetic structure of the Mormon metalmark butterfly *Apodemia mormo* in British Columbia, detecting a high degree of spatial genetic structure within the population, despite a small geographic range (< 20 km). At a larger scale, Wolf et al. (2004) effectively used AFLPs to assess genetic structure of *Rhododendron ferrugineum*, a subalpine shrub, sampled from sites with distances of 4 km to

more than 1028 km between them. Thus, AFLPs are highly appropriate markers for a multi-scale study of population genetic structure such as mine.

Chapter 2: Materials and Methods

2.1 Collection of larvae

Second-instar *W. smithii* larvae were collected by G. Rasic in Algonquin Provincial Park (Ontario, Canada; UTM: 17N 687337E 5046853N; Figure 2.1) in August 2009 at five nested spatial scales, which included leaf, plant, cluster of plants, peatland, and regions (called 'systems') of peatlands (Figures 2.2 and 2.3). Larvae, and not adults, were sampled because capture of adults was impractical due to their very small body size and the difficulty of attracting them to traps (Krawchuk & Taylor 2003; Rasic 2011). Furthermore, larvae are associated with individual pitcher plant leaves and plants, while flying adults cannot be so attributed.

The sampled peatlands were located within a forested matrix containing coniferous forests dominated by pines (*Pinus spp.*), poplar (*Populus sp.*), and white birch (*Betula papyrifera*), and deciduous forests dominated by sugar maple (*Acer saccharum*) and beech (*Fagus grandifolia*). The peatlands consisted of both true bogs and poor fens. Bogs are nutrient-poor, acidic peatlands dominated by *Sphagnum* mosses; their only source of water is through precipitation (Spitzer & Danks 2006; Mitsch & Gosselink 2007; Keddy 2010). Poor fens are also acidic, but have more sedge cover than bogs, and receive some minerals from groundwater (Mitsch & Gosselink 2007).

Two separate peatland systems within Algonquin Provincial Park, located 26 km apart, were sampled by G. Rasic (Figure 2.1). From each system, four neighbouring peatlands, which were 0.2-7.0 km apart, were sampled (Figure 2.2). Therefore eight peatlands were included in the study. Within every peatland, three clusters of pitcher plants were arbitrarily selected, with each containing at least ten plants. Then, three plants were arbitrarily selected within each cluster, and three leaves were selected per plant (Figure 2.3). All larvae were pipetted out of each selected leaf, placed in absolute ethanol in 1.7 ml microcentrifuge tubes, and stored at -20°C. Using a high-accuracy Global Positioning System (Trimble GeoXH) receiver, the sites of all sampled pitcher plants were documented by G. Rasic (2011) to within 1 m precision.

During sampling, larvae of both *W. smithii* and the pitcher plant midge, *M. knabi*, were collected simultaneously, and larvae of both species from a single leaf were stored together in one tube of ethanol. I separated out the *W. smithii* larvae, which are easily distinguishable from *M. knabi* larvae by virtue of their enlarged heads and thoraxes, as well as prominent lateral hairs. Furthermore, *W. smithii* is the only living mosquito that would be found within the leaves of *S. purpurea* in my study area (Bradshaw & Lounibos 1977). Nonetheless, I discarded any larvae that were degraded and therefore could not be unequivocally identified as *W. smithii.*

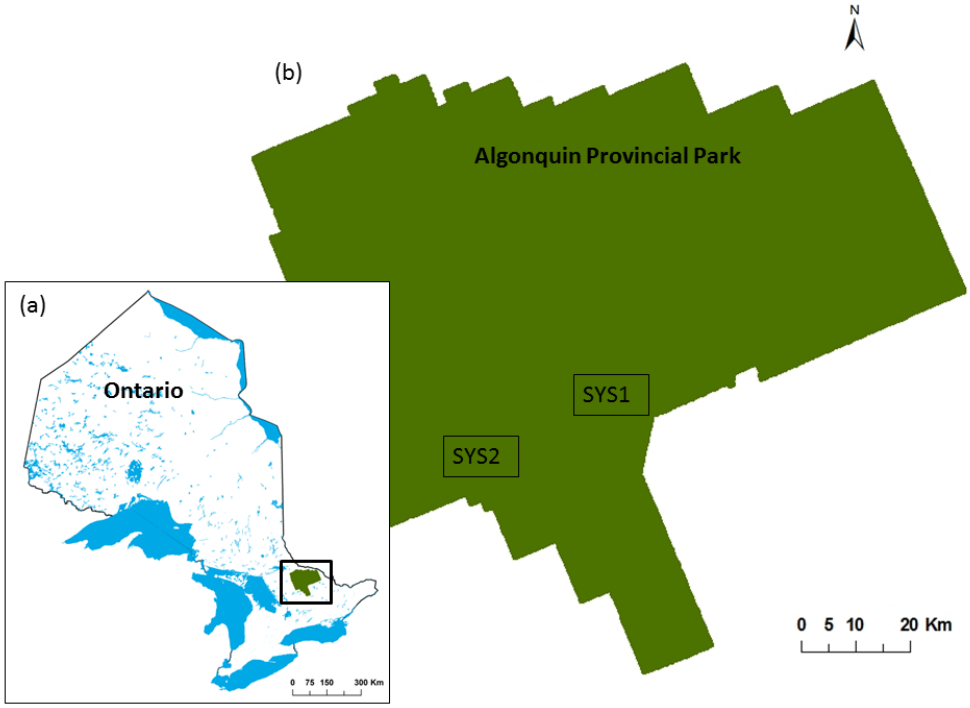

Figure 2.1 The location of two systems of peatlands in Algonquin Provincial Park, Ontario, Canada, from which larvae of the pitcher plant mosquito, *Wyeomyia smithii*, were obtained. (a) The location of the Park within Ontario is shown, in green and outlined with a black box. (b) Locations of the two systems (SYS2 and SYS1) within the Park are shown, each outlined with a black box. Both maps were created using ArcGIS 10.0 (Redlands, CA).

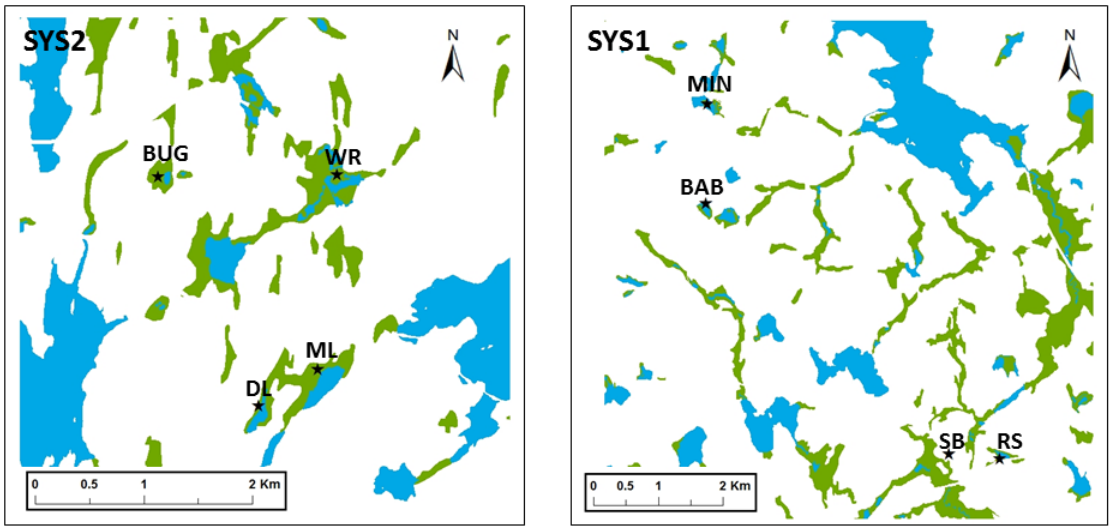

Figure 2.2 The locations of four peatlands within each of two peatland systems (SYS2 and SYS1) in Algonquin Provincial Park, Ontario, Canada from which larvae of the pitcher plant mosquito, *Wyeomyia smithii*, were obtained. Water bodies are shown in blue shading, and wetlands are shown in green shading. Each star indicates a sampled site (BUG=Buggy; WR=West Rose; DL=Dizzy Lake; ML=Mizzy Lake; MIN=Minor Lake; BAB=Bab Lake; SB=Spruce Bog; RS=Roadside). Both maps were created using ArcGIS 10.0 (Redlands, CA).

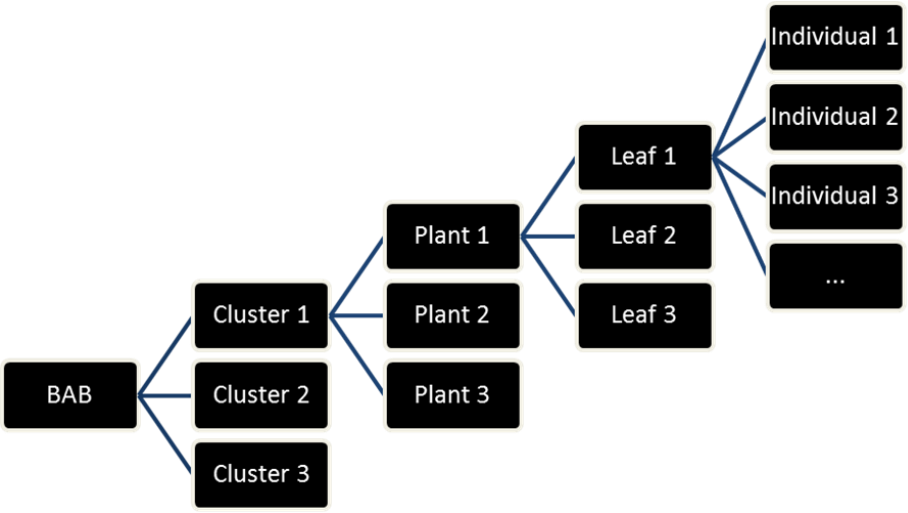

Figure 2.3 Hierarchical sampling scheme for larvae of *Wyeomyia smithii*, using BAB peatland as an example. Within each peatland, three clusters were selected, where a cluster was considered an aggregation of at least ten plants. Within each cluster, three plants were selected. Within each plant, three leaves were selected, and larvae were pipetted from each of these three leaves. All larvae within each of the selected leaves were removed. Design and execution of sampling were completed by G. Rasic in August 2009.

2.2 DNA extraction and AFLP protocol

To extract high molecular weight DNA that was free of contaminants, I used a QIAgen DNeasy® Blood & Tissue Kit (QIAgen, Germantown, Maryland), which enables the purification of total DNA from insects. DNA from each larva was extracted individually, and eluted in 100 µL of QIAgen elution buffer (buffer 'EB'). To increase its concentration, DNA from each larva was then precipitated using a standard ethanol precipitation, and dissolved in 45 µL water.

AFLP fragments were generated using standard approaches (Vos et al. 1995), with negative (water) controls included at each stage of the protocol to ensure that contamination had not occurred. Briefly, for each individual sample, genomic DNA was digested with EcoRI and MseI restriction enzymes (New England Biolabs, Ipswich, MA), and double-stranded adaptors (Applied Biosystems, Foster City, CA; Table 2.1) were ligated to the resulting

fragments. The ligation of adaptors to restriction fragments generates a template for the subsequent polymerase chain reactions (PCR).

The fragments were then filtered based on terminal sequences and amplified using two successive PCR reactions (pre-selective and selective PCRs). Thermal cycling and chemistry parameters differed for pre-selective and selective PCR amplifications (Tables 2.2-2.5). To verify that successful selective amplification had occurred at each PCR stage, 5.0 µL of PCR product was run on a 1.5% agarose gel. Each successful selective PCR amplification reaction resulted in a series of distinct bands from 100-500 base pairs in length. In the final selective PCR amplification, one primer from each pair was fluorescently labelled, so that the resulting PCR products could be detected on an automated DNA analyzer. Selective PCR products were electrophoresed at high resolution and their sizes determined using a 3730XL DNA Analyzer (Applied Biosystems).

I initially tested 24 selective primer pair combinations by using each of them to genotype 15 individuals, and then chose only the following 4 pairs based on reproducibility, number of bands produced, and minimal background noise: E*co*RI-AAC/M*se*I-CAG, E*co*RI-AAC/M*se*I-CAT, E*co*RI-ACG/M*se*I-CTA, and E*co*RI-AGC/M*se*I-CTA.

Table 2.1 Oligonucleotide sequences of adaptors and pre-selective primers used in the AFLP protocol, for the genomic DNA of samples of the pitcher plant mosquito, *Wyeomyia smithii*. EcoRI Adaptor 1 and EcoRI Adaptor 2 were annealed to generate a double-stranded adaptor. Likewise for MseI Adaptor 1 and MseI Adaptor 2.

Class	Oligo name	Sequence (5'-3')
Adaptors	EcoRI Adaptor 1	CTCGTAGACTGCGTACC
	EcoRI Adaptor 2	AATTGGTACGCAGTCTAC
	MseI Adaptor 1	GACGATGAGTCCTGAG
	MseI Adaptor 2	TACTCAGGACTCAT
Pre-selective primers	EcoRI Primer	GACTGCGTACCAATTCA
	MseI Primer	GATGAGTCCTGAGTAAC

Table 2.2 Pre-selective PCR amplification reaction of the AFLP protocol, for *Wyeomyia smithii*.

Reagent	Final reaction conditions	Volume (µL)
Milli-Q H_20		3.05
10× PCR buffer		2.00
Betaine	1.05 M	7.00
dNTPs	0.25 mM	0.50
$MgCl_2$	1.56 mM	1.25
EcoRI pre-selective primer	0.5 µM	1.00
MseI pre-selective primer	0.5 µM	1.00
Taq polymerase	0.05 U	0.20
Restriction-ligation DNA		4.00
Total		20.00

Table 2.3 Selective PCR amplification reaction of the AFLP protocol, for *Wyeomyia smithii*.

Reagent	Final reaction conditions	Volume (µL)
Milli-Q H_20		8.80
10× PCR buffer		2.00
dNTPs	0.25 mM	0.50
$MgCl_2$	4.38 mM	3.50
EcoRI selective primer	0.5 µM	1.00
MseI selective primer	0.5 µM	1.00
Taq polymerase	0.05 U	0.20
Pre-selective PCR product		3.00
Total		20.00

Table 2.4 AFLP thermal cycler parameters for the pre-selective amplification reaction, for *Wyeomyia smithii*.

Step	Temperature (˚C)	Time	Number of cycles
1	72	2 min	1
2	94	20 s	
	56	30 s	25
	72	2 min	
3	60	30 min	1
4	4	-	1

Table 2.5 AFLP thermal cycler parameters for the selective amplification reaction, for *Wyeomyia smithii*.

Step	Temperature (°C)	Time	Number of step cycles
1	94	2 min	1
2	94	20 s	
	Annealing temperature*	30 s	10
	72	2 min	
3	60	30 min	1
4	4	end	1

*Step 2 consists of ten touch-down cycles, starting with 20 s (hold at 94°C), 30 s (hold at 66°C), 2 min (hold at 72°C), and lowering the annealing temperature by 1°C each cycle.

2.3 Genotype scoring

I used the genotype scoring program GeneMapper v. 4.0 (Applied Biosystems) to visualize AFLP profiles (i.e., the complete set of AFLP peaks or DNA fragments) for each sample (Meudt & Clarke 2007). I identified loci (referred to as ‘bins’ in GeneMapper) between 100 and 500 base pairs (bp) in size, with all bins being 1 bp wide. Fragments smaller than 100 bp were disregarded to reduce the prevalence of size homoplasy (Vekemans et al. 2002). I

checked bins manually and deleted any bins with overlapping fragments. Additionally, all AFLP profiles were checked manually and were removed if the sample failed to amplify.

I left the AFLP peak height data un-normalized in GeneMapper, and then normalized and scored them in AFLPScore v. 2.15 (Whitlock et al. 2008). AFLPScore interprets PCR-product fluorescence intensity data (i.e., peak heights) generated from DNA analyzers to create presence-absence (1-0) phenotype tables based on locus-selection and phenotype-calling thresholds. AFLPScore objectively determines the optimal thresholds to minimize genotyping error based on comparison of replicate samples. Thus, I first applied thresholds to determine which loci were fit for inclusion in analysis (‘locus selection threshold’), then determined the phenotype of each individual (i.e., band presence or absence) using ‘phenotype calling thresholds’. For each selective primer pair, different locus-selection thresholds were used such that only loci with peak height values equal to or above the threshold were retained for analysis (Table 2.6). I also used absolute phenotype-calling thresholds for each primer combination, so that for each individual at a given selective primer pair, peaks with a height equal to or greater than this value were scored as a '1' (presence) phenotype, and those with a lower peak height were scored as a '0' (absence) phenotype.

For each primer set, AFLPScore computed mismatch error rates based on the percentage of differences in phenotype calls between replicates of duplicated samples (which were generated from separate aliquots of the same DNA extraction that were subjected to independent runs of the entire AFLP protocol). Running randomly selected samples through the whole genotyping process is an accurate way of approximating error rate, because these replicates accrue the effects of all potential error sources (Bonin et al. 2004). The recommended number of replicates is 5 to 10% of the total sample size (Bonin et al. 2004). The number of replicates I used for each primer set ranged from approximately 6 to 9% (Table 2.7).

Table 2.6 For the four final selective primer combinations, AFLP phenotype scoring results generated by AFLPScore v. 2.15 (Whitlock et al. 2008) for all *Wyeomyia smithii* samples. rfu are 'relative fluorescence units.'

Selective primer pair	Locus-selection threshold (rfu)	Phenotype-selection threshold (rfu)	Number of initial loci	Number of loci retained	Final mismatch error rate (%)
E*co*RI-AAC / M*se*I-CAG	2000	1000	57	34	4.0
E*co*RI-AAC / M*se*I-CAT	2000	1200	58	34	4.0
E*co*RI-ACG / M*se*I-CTA	8000	3000	46	15	4.0
E*co*RI-AGC / M*se*I-CTA	4000	2000	62	34	4.5
Total			223	117	

Table 2.7 Replicates used for each selective primer set in order to estimate mismatch error rate in AFLPScore v. 2.15 (Whitlock et al. 2008), for the genotyping of *Wyeomyia smithii*.

Selective primer pair	Total number of samples	Number of replicates
E*co*RI-AAC / M*se*I-CAG	558	35
E*co*RI-AAC / M*se*I-CAT	577	47
E*co*RI-ACG / M*se*I-CTA	575	40
E*co*RI-AGC / M*se*I-CTA	570	39

In AFLPScore, I chose thresholds that resulted in error rates within the recommended range of 2-5% while retaining the greatest numbers of loci for each primer set (Meudt & Clarke 2007). Ultimately, 117 informative and reliable markers were generated in total (error rate <5%).

2.4 Data analysis

Based on the locus presence-absence phenotypes generated using AFLPScore, I estimated genetic diversity, population genetic structure, and relatedness coefficients using AFLP-SURV v. 1.0 (Vekemans et al. 2002). I used the script AFLPdat (Ehrich 2006) in the statistical software package R (R Development Core Team 2012), to arrange input files for AFLP-SURV. In AFLP-SURV, assuming Hardy-Weinberg genotype proportions, I used the Bayesian method with non-uniform prior distribution to estimate allele frequencies.

As measures of genetic diversity, I estimated the proportion of loci polymorphic at the 5% level (PPL) and unbiased expected heterozygosity (H_j) following the methods of Lynch & Milligan (1994) as implemented in AFLP-SURV. These estimates were obtained using both clusters and peatlands as the units of analysis (i.e., treating clusters or peatlands as separate 'populations').

I also estimated F_{ST} values in AFLP-SURV. A measure of genetic differentiation, F_{ST} is used extensively in population and evolutionary genetics to describe genetic structure, and can be defined as the correlation of randomly chosen alleles within a population relative to that among populations (Wright 1965; Holsinger & Weir 2009). F_{ST} can be estimated for two (i.e., 'pairwise') or more populations and ranges from 0 to 1, with small values indicating that allele frequency distributions of the populations being compared are similar, and large values indicating that the populations are genetically differentiated (Holsinger & Weir 2009). Permutation tests are typically performed to estimate standard errors and determine whether estimated values of F_{ST} are greater than zero, in which case populations are considered significantly differentiated (i.e., they are not panmictic). I estimated F_{ST} for all sampled populations simultaneously (i.e., a single 'global' estimate of F_{ST}) as well as between pairs of populations. This was done using both clusters and peatlands as the units of analysis. Statistical significance of F_{ST} values was assessed based on 1000 permutations.

I tested for isolation-by-distance (IBD), or a significant correlation of pairwise F_{ST} values and pairwise geographic distance (m), using the Mantel test (Mantel 1967) in GENALEX v. 6.41 (Peakall & Smouse 2006). Significance of Mantel tests was assessed using 999 permutations. The IBD analysis was performed using both clusters and peatlands as the units

of analysis, with geographic distances measured between the centroids of pairs of clusters and peatlands, respectively.

To examine how genetic variation was partitioned across each spatial scale, I employed the hierarchical analysis of molecular variance (AMOVA) using the package HIERFSTAT for the statistical software R (Goudet 2005). HIERFSTAT estimates variance components and hierarchical *F*-statistics (analogous to F_{ST}) for any number of nested levels, and therefore is highly appropriate for the hierarchical nature of the pitcher plant system.

I estimated the relatedness of each pair of larvae in my data set according to the method of Hardy (2003) using AFLP-SURV. This method can use data from dominant genetic markers, such as AFLPs, to estimate pairwise relatedness between individuals (Hardy 2003). The relatedness coefficients indicate the degree of genetic similarity between individuals and are conceptually related to kinship coefficients from pedigrees. However, because the relatedness coefficients measure the genetic similarity of a pair of individuals relative to the average genetic similarity of all individuals from a 'reference population' (in my case, the sample of all individuals in the data set), they are not numerically equivalent to pedigree-based kinship coefficients and can take on negative values (Hardy 2003). A negative value indicates that the pair of individuals under consideration is less related, on average, than most pairs of individuals in the reference population. Using the relatedness estimates, I calculated the mean relatedness for pairs of individuals at each of the following scales within each peatland: (i) pairs of individuals from the same leaf, (ii) pairs of individuals from different leaves within the same plant, (iii) pairs of individuals from different plants within the same cluster, and (iv) pairs of individuals from different clusters within the same peatland.

To assess whether female *W. smithii* were more likely to lay multiple eggs in a single leaf (i.e., to 'clump' their eggs) when pitcher plant availability was low, I examined the relationship between a measure of the clumping of related larvae at the leaf scale and plant density, among peatlands, using a generalized linear model (GLM), with normal distribution and identity link. The GLM was analyzed in JMP® v 8.0.1. software (SAS Institute Inc., Cary, NC). To obtain a measure of clumping of related larvae at the leaf scale, I could not simply use mean relatedness at the leaf scale, because relatedness across all scales differed among the peatlands. Therefore, to attain a measure of 'clumping' for each peatland, I

calculated the differences between the mean pairwise relatedness of individuals from leaves of the same plant and the mean pairwise relatedness of individuals from different plants and different clusters (i.e., at the two broadest scales of the spatial hierarchy). This was a measure, for each peatland, of how much more related individuals in the same leaf were relative to individuals sampled at the broader spatial scales. The independent variables in the GLM were the natural logs of peatland size and pitcher plant density in each peatland, which were reported by Rasic & Keyghobadi (2012b), and an interaction term was included.

Chapter 3: Results

I successfully scored 597 individuals at 117 AFLP loci generated by four selective primer pairs. Thus, my final AFLP data set consisted of 597 individuals scored for presence/absence (indicated by 1 or 0, respectively) of 117 loci (i.e., a 597 X 117 matrix). This data matrix is available from the author upon request. The mean number of individuals genotyped per leaf was 3.5. Final mismatch error rates for the primer pairs ranged from 4.0 to 4.5%. My final data set consisted of 314 individuals from System 1, and 283 individuals from System 2.

Using peatlands as the unit of analysis, the proportion of polymorphic loci (PPL) within populations ranged from 25.6 to 44.4%, and the expected heterozygosity (H_j) ranged from 0.116 to 0.159, with West Rose peatland (WR) showing the highest levels of genetic diversity (Table 3.1).

Table 3.1 Values of genetic diversity for pitcher plant mosquito, *Wyeomyia smithii*, larvae collected from eight peatlands in Algonquin Provincial Park, where the peatland is the unit of analysis.

Site/Population	*N*	PPL (%)	H_j (±SE)
BAB	66	32.5	0.130 (0.017)
BUG	73	29.9	0.116 (0.016)
DL	65	36.8	0.140 (0.017)
MIN	75	25.6	0.122 (0.016)
ML	81	33.3	0.127 (0.016)
RS	82	29.1	0.117 (0.016)
SB	91	31.6	0.122 (0.016)
WR	64	44.4	0.159 (0.017)

N is the number of analyzed samples; PPL is the proportion of polymorphic loci (at the 5% level); H_j is the expected heterozygosity.
BUG=Buggy; WR=West Rose; DL=Dizzy Lake; ML=Mizzy Lake; MIN=Minor Lake; BAB=Bab Lake; SB=Spruce Bog; RS=Roadside.

Using clusters as the unit of analysis, the proportion of polymorphic loci (PPL) ranged from 14.5 to 57.3%, and the expected heterozygosity (H_j) ranged from 0.063 to 0.184, with Cluster 3 of West Rose peatland (WR-3) showing the highest levels of genetic diversity (Table 3.2).

Table 3.2 Values of genetic diversity for pitcher plant mosquito, *Wyeomyia smithii,* larvae collected from Algonquin Provincial Park, where cluster is the unit of analysis. Larvae were sampled from three clusters within each peatland.

Site/Population	*N*	PPL (%)	H_j (±SE)
BAB-1	22	29.1	0.097 (0.014)
BAB-2	19	41.9	0.164 (0.017)
BAB-3	25	33.3	0.126 (0.017)
BUG-1	18	32.5	0.120 (0.017)
BUG-2	31	24.8	0.086 (0.013)
BUG-3	24	35.9	0.142 (0.017)
DL-1	21	41.9	0.156 (0.018)
DL-2	14	41.9	0.169 (0.018)
DL-3	30	27.4	0.116 (0.016)
MIN-1	17	26.5	0.106 (0.016)
MIN-2	28	29.9	0.124 (0.016)
MIN-3	30	30.8	0.134 (0.017)
ML-1	29	35.9	0.134 (0.017)
ML-2	30	40.2	0.146 (0.017)
ML-3	22	14.5	0.063 (0.012)
RS-1	30	31.6	0.108 (0.015)
RS-2	22	38.5	0.138 (0.018)
RS-3	30	29.9	0.106 (0.016)
SB-1	30	38.5	0.141 (0.017)
SB-2	31	22.2	0.095 (0.015)
SB-3	30	32.5	0.130 (0.018)
WR-1	28	39.3	0.145 (0.017)
WR-2	18	41.0	0.162 (0.018)
WR-3	18	57.3	0.184 (0.015)

N is the number of analyzed samples; PPL is the proportion of polymorphic loci at 5% level; H_j is the expected heterozygosity.
The number after each abbreviated peatland name is the number of that cluster.
BUG=Buggy; WR=West Rose; DL=Dizzy Lake; ML=Mizzy Lake; MIN=Minor Lake; BAB=Bab Lake; SB=Spruce Bog; RS=Roadside.

Levels of genetic differentiation among peatlands and clusters were low, with global F_{ST} among peatlands being only 0.0165 and global F_{ST} among clusters being only 0.0502 (Table 3.3). Neither of these F_{ST} estimates was significantly greater than zero (both $P>0.05$). Pairwise F_{ST} values between peatlands ranged from 0.0005 to 0.064, and between clusters ranged from zero to 0.2966. Samples from West Rose peatland were the most highly differentiated; for example, using peatlands as the unit of analysis, the average pairwise F_{ST}

for West Rose peatland (compared to all other peatlands) was 0.038, while average pairwise F_{ST} between all other pairs of peatlands was only 0.014. Because samples from West Rose peatland (in System 2) stood out as being highly genetically differentiated from samples from other peatlands, the isolation-by-distance (IBD) and HIERFSTAT analyses were conducted both including and not including this peatland.

Table 3.3 Global estimates of genetic differentiation (F_{ST}) among populations of the pitcher plant mosquito, *Wyeomyia smithii*, from Algonquin Provincial Park.

Unit of analysis (i.e. population)	F_{ST} (±SE)
Peatland	0.0165 (0.1557)
Cluster	0.0502 (0.1989)

Standard error of F_{ST} was assessed based on 1000 permutations.

Using peatlands as the unit of analysis, there was no significant pattern of IBD, that is a correlation between pairwise genetic differentiation (F_{ST}) and geographic distance, when West Rose peatland was included in the analysis (P=0.233; Figure 3.1). However, IBD was significant among peatlands from both systems when West Rose peatland was excluded (P=0.010; Figure 3.2). Within each system, excluding West Rose peatland, there was no significant pattern of IBD (P=0.204 for System 1; P=0.664 for System 2). Additionally, when clusters were the unit of analysis, IBD was not significant either between systems, or within each system, whether West Rose was included or not (all P>0.05; Figures 3.3 and 3.4).

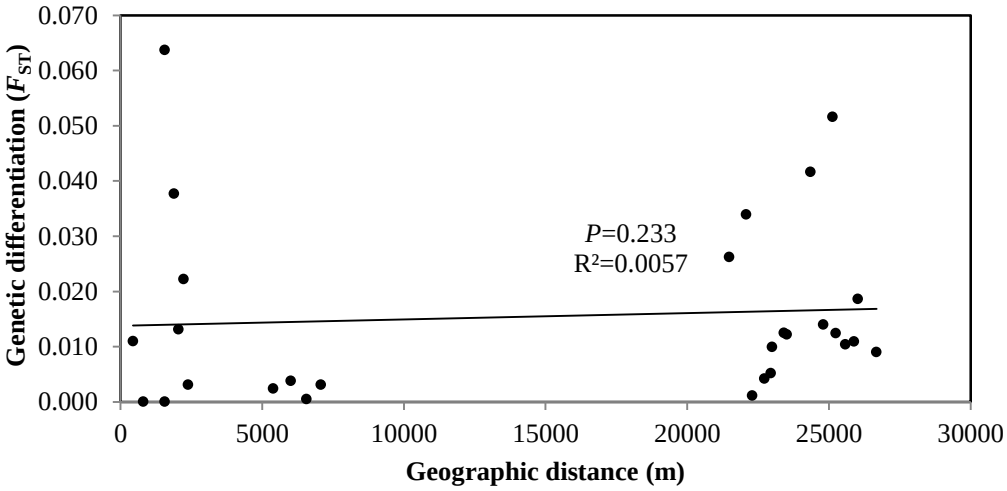

Figure 3.1 Isolation-by-distance for pairs of populations of the pitcher plant mosquito, *Wyeomyia smithii*, sampled from eight peatlands, including West Rose peatland, in Algonquin Provincial Park, Canada. Significance was assessed using Mantel test in GENALEX v. 6.41 (Peakall & Smouse 2006). The peatland is the unit of analysis, and each point represents a pair of peatlands.

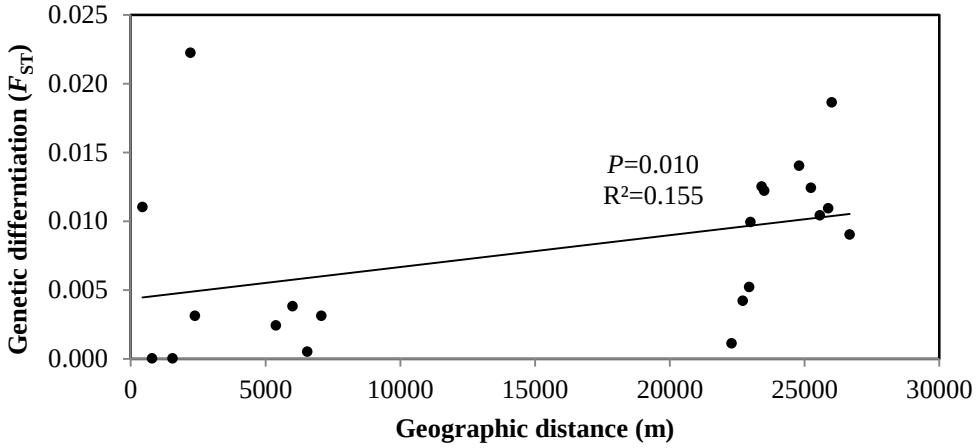

Figure 3.2 Isolation-by-distance among populations of the pitcher plant mosquito, *Wyeomyia smithii*, sampled from seven peatlands, not including West Rose peatland, in Algonquin Provincial Park, Canada. Significance was assessed using Mantel test in GENALEX v. 6.41 (Peakall & Smouse 2006). The peatland is the unit of analysis and each point represents a pair of peatlands.

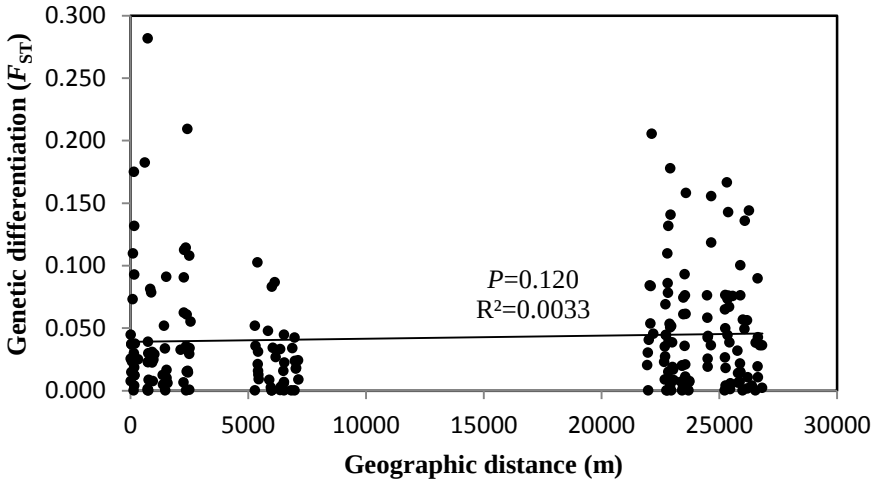

Figure 3.3 Isolation-by-distance for pairs of clusters of the pitcher plant mosquito, *Wyeomyia smithii*, sampled from seven peatlands, excluding West Rose peatland. Significance was assessed using Mantel test in GENALEX v. 6.41 (Peakall & Smouse 2006). The cluster is the unit of analysis, and each point represents a pair of clusters.

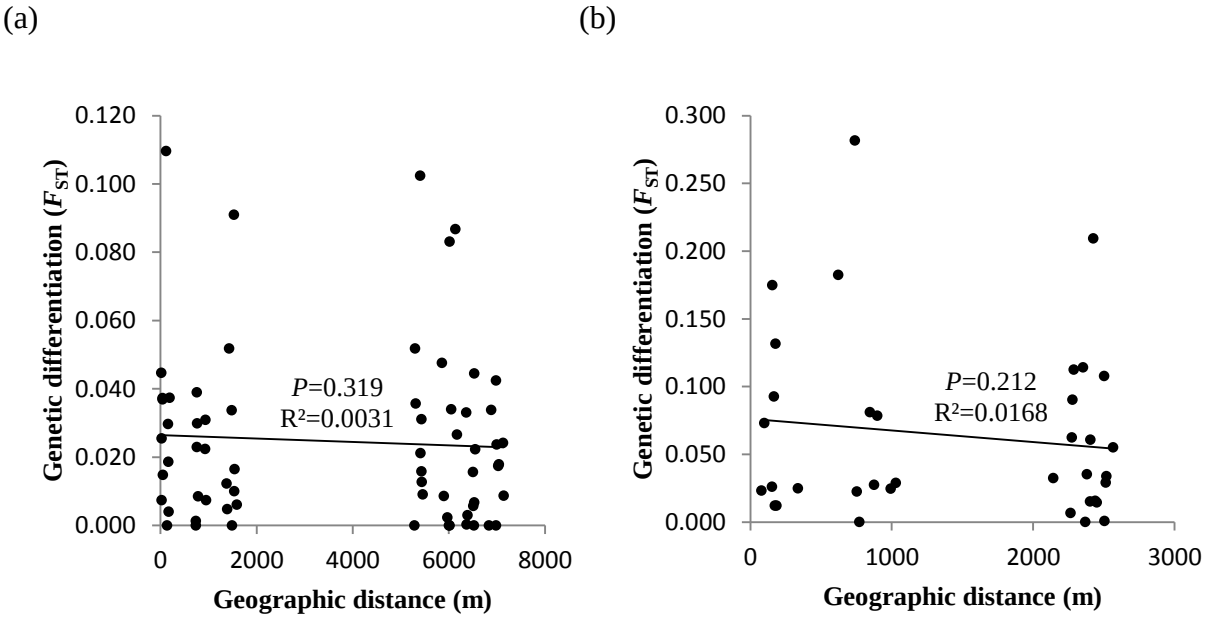

Figure 3.4 Isolation-by-distance for pairs of clusters in (a) System 1 (MIN, BAB, SB, RS) and (b) System 2 (BUG, DL, ML). The cluster is the unit of analysis and each point represents a pair of clusters.(BUG=Buggy; DL=Dizzy Lake; ML=Mizzy Lake; MIN=Minor Lake; BAB=Bab Lake; SB=Spruce Bog; RS=Roadside.)

When West Rose peatland was included in the analysis, HIERFSTAT revealed that there was significant differentiation among leaves on the same plant (i.e., a high level of variance could be attributed to this scale), as well as among peatlands within the same system (Table 3.4). However, there was no significant differentiation between the two systems (Table 3.4). When West Rose peatland was not included in the HIERFSTAT analysis, there was no significant differentiation at any scale of the spatial hierarchy except among leaves within the same plant (Table 3.5).

Analyzing the two systems separately, and also excluding West Rose peatland, a difference between the two systems was found at the plant scale: in System 1, genetic differentiation among plants within the same cluster was not significant (P=0.775), but it was significant in System 2 (P=0.01). For both systems, there was significant differentiation among leaves within the same plant (Table 3.6; P=0.003 for System 1; P=0.001 for System 2).

Table 3.4 Hierarchical analysis of molecular variance (AMOVA) in *Wyeomyia smithii* for all peatlands in both Systems 1 and 2, including West Rose peatland. The output from HIERFSTAT (Goudet 2005) displays *F*-statistics (measures of genetic structuring) at each scale. The value in a given cell indicates differentiation among units of the corresponding column within units of the corresponding row. As an example, the *F*-statistic measuring differentiation of clusters within a peatland is -0.017. The most relevant values on which to focus are boxed. Significance of *F*-statistics was computed using 1000 permutations, and those values significantly greater than zero are bolded.

Scale	System	Peatland	Cluster	Plant	Leaf
Total	0.011	0.017	0.000	0.048	0.337
System		**0.006**	-0.011	0.037	0.329
Peatland			-0.017	0.032	0.326
Cluster				0.048	0.337
Plant					**0.303**

Table 3.5 Hierarchical analysis of molecular variance (AMOVA) in *Wyeomyia smithii* for peatlands in both Systems 1 and 2, excluding West Rose peatland. The output from HIERFSTAT (Goudet 2005) displays *F*-statistics (measures of genetic structuring) at each scale. The value in a given cell indicates differentiation among units of the corresponding column within units of the corresponding row. As an example, the *F*-statistic measuring differentiation of peatlands in systems is -0.005. The most relevant values on which to focus are boxed. Significance of *F*-statistics was computed using 1000 permutations, and those values significantly greater than zero are bolded.

Scale	System	Peatland	Cluster	Plant	Leaf
Total	0.011	0.006	-0.002	-0.003	0.337
System		-0.005	-0.013	-0.014	0.329
Peatland			-0.008	-0.008	0.333
Cluster				-0.001	0.338
Plant					**0.339**

Table 3.6 Hierarchical analysis of molecular variance (AMOVA) in *Wyeomyia smithii* for each system ('sys') of peatlands, excluding West Rose peatland. The output from HIERFSTAT (Goudet 2005) displays *F*-statistics (measures of genetic structuring) at each scale. The value in a given cell indicates differentiation among units of the corresponding column within units of the corresponding row. As an example, the *F*-statistic measuring differentiation of clusters within a peatland in System 2 is 0.010. The most relevant values on which to focus are boxed. Significance of *F*-statistics was computed using 1000 permutations, and values significantly greater than zero are bolded.

Scale	Peatland		Cluster		Plant		Leaf	
	Sys1	Sys2	Sys1	Sys2	Sys1	Sys2	Sys1	Sys2
Total	-0.002	-0.011	-0.02	0.000	-0.065	0.058	0.309	0.356
Peatland			-0.019	0.010	-0.063	0.068	0.31	0.363
Cluster					-0.044	**0.059**	0.323	0.357
Plant							**0.351**	**0.317**

The relatedness estimates generated by AFLP-SURV indicated a general trend of higher relatedness values between individuals sampled from the same leaf, compared to pairs of individuals sampled from different leaves, plants or clusters (Figures 3.5 and 3.6). This pattern was consistent across almost all peatlands. In most peatlands, relatedness values dropped substantially between the leaf and plant scales; that is, individuals from different leaves of the same plant had much lower relatedness than individuals sampled from the same leaf. In two peatlands of System 2, Dizzy Lake (DL) and Mizzy Lake (ML), mean relatedness of pairs of individuals increased very slightly between the leaf and plant scale, but then dropped at the higher scales. Overall, across all scales, relatedness of individuals in West Rose peatland (WR) was lower than relatedness of individuals in all other peatlands. Nonetheless, West Rose showed a very steep change in relatedness between the leaf and plant scales, indicating a high degree of aggregation of the most closely related individuals within leaves of the same plant.

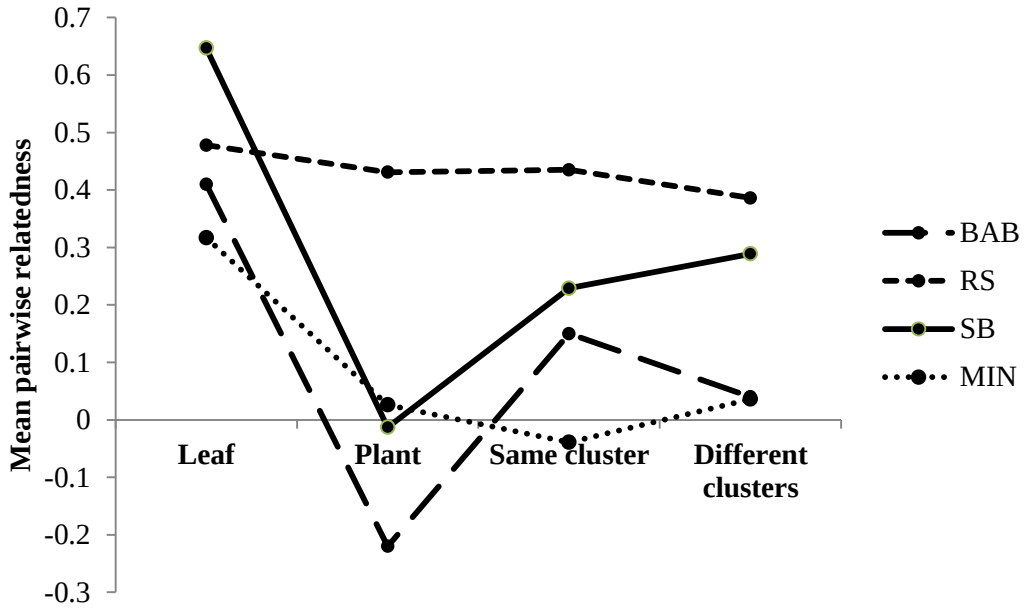

Figure 3.5 Mean relatedness values between pairs of individual larvae of *Wyeomyia smithii* collected from Algonquin Provincial Park (System 1). For each peatland within System 1 (BAB=Bab Lake, RS=Roadside, SB=Spruce Bog, and MIN=Minor Lake), the mean pairwise relatedness is shown for pairs of individuals at various scales: Leaf = pairs of individuals within the same leaf, Plant = pairs of individuals from different leaves within the same plant, Same cluster = pairs of individuals from different plants within the same cluster, and Different clusters = pairs of individuals from different clusters.

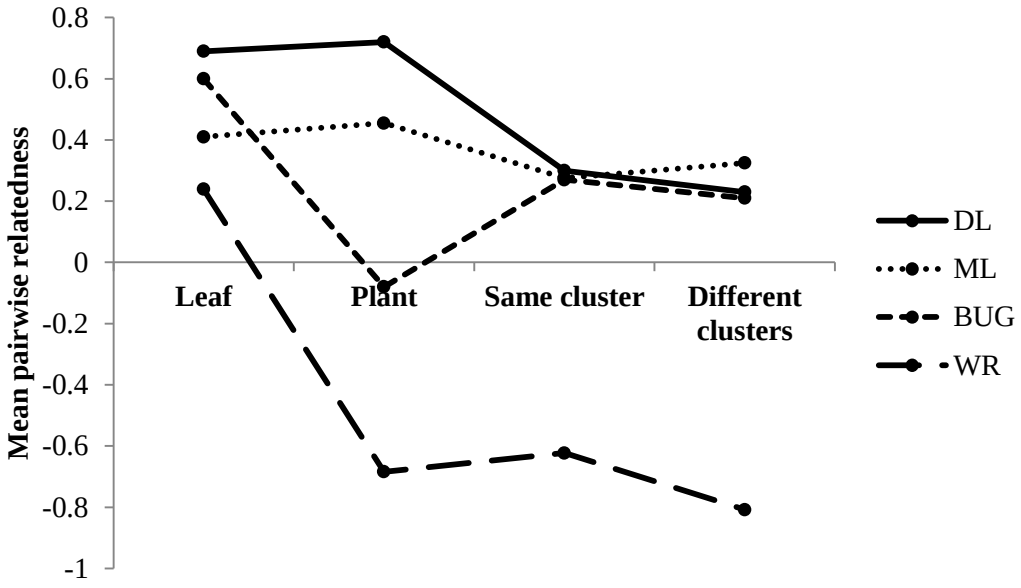

Figure 3.6 Mean relatedness values between pairs of individual larvae of *Wyeomyia smithii* collected from Algonquin Provincial Park (System 2). For each peatland within System 2 (DL=Dizzy Lake, ML=Mizzy Lake, BUG=Buggy, and WR=West Rose), the mean pairwise relatedness is shown for pairs of individuals at various scales: Leaf = pairs of individuals within the same leaf, Plant = pairs of individuals from different leaves within the same plant, Same cluster = pairs of individuals from different plants within the same cluster, and Different clusters = pairs of individuals from different clusters.

Examining the effects of peatland size and pitcher plant density on the degree of aggregation (clumping) of related larvae within leaves, I first removed the interaction term between peatland size and plant density because the generalized linear model (GLM) without the interaction had a better fit (i.e., a much lower Akaike Information Criterion, AIC_c) than did the model with the interaction (AIC_c with interaction = 29.05 and AIC_c without interaction = 14.38). The resulting GLM revealed a significant negative relationship between pitcher plant density and clumping of related larvae. The model was significant overall (P=0.022), as was the effect of pitcher plant density (P=0.0097; Figure 3.7). The effect of peatland size was not significant (P=0.85). Thus, larvae within individual leaves had high relatedness, relative to relatedness of larvae from different plants or clusters, in peatlands with low pitcher plant density. However, this relationship appeared to be largely driven by a single peatland, West

Rose, with very low plant density and very high egg clumping index (Figure 3.7). When West Rose peatland was removed from the GLM, neither the overall model (*P*=0.88) nor the effect of pitcher plant density (*P*=0.62) was significant (Figure 3.8).

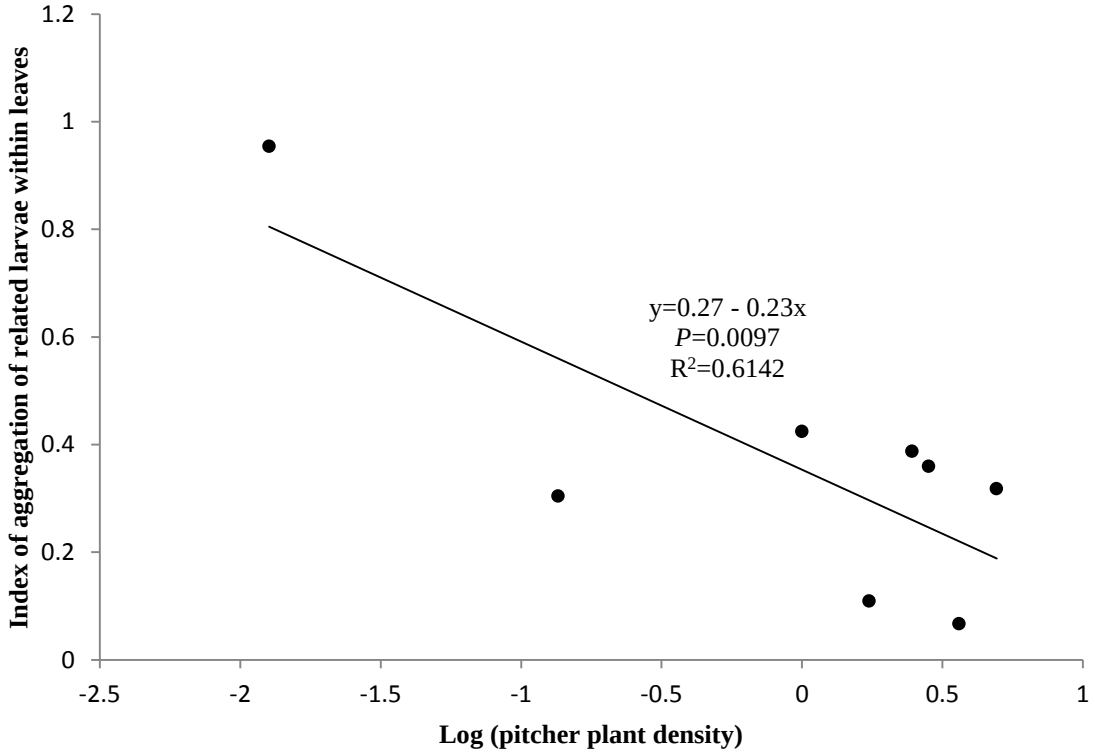

Figure 3.7 Relationship between an index of aggregation (i.e., clumping) of related larvae of the pitcher plant mosquito, *Wyeomyia smithii*, within leaves and pitcher plant density. Data are for eight peatlands from Algonquin Provincial Park (including West Rose peatland). The y-axis shows the mean pairwise relatedness of individual larvae sampled from different plants and clusters, subtracted from the mean pairwise relatedness of individual larvae sampled from the same leaf. The x-axis shows the natural logarithm of pitcher plant density in each peatland. Slope and intercept estimates, and P-value, are from a Generalized Linear Model with pitcher plant density and peatland area (both log transformed) as independent variables.

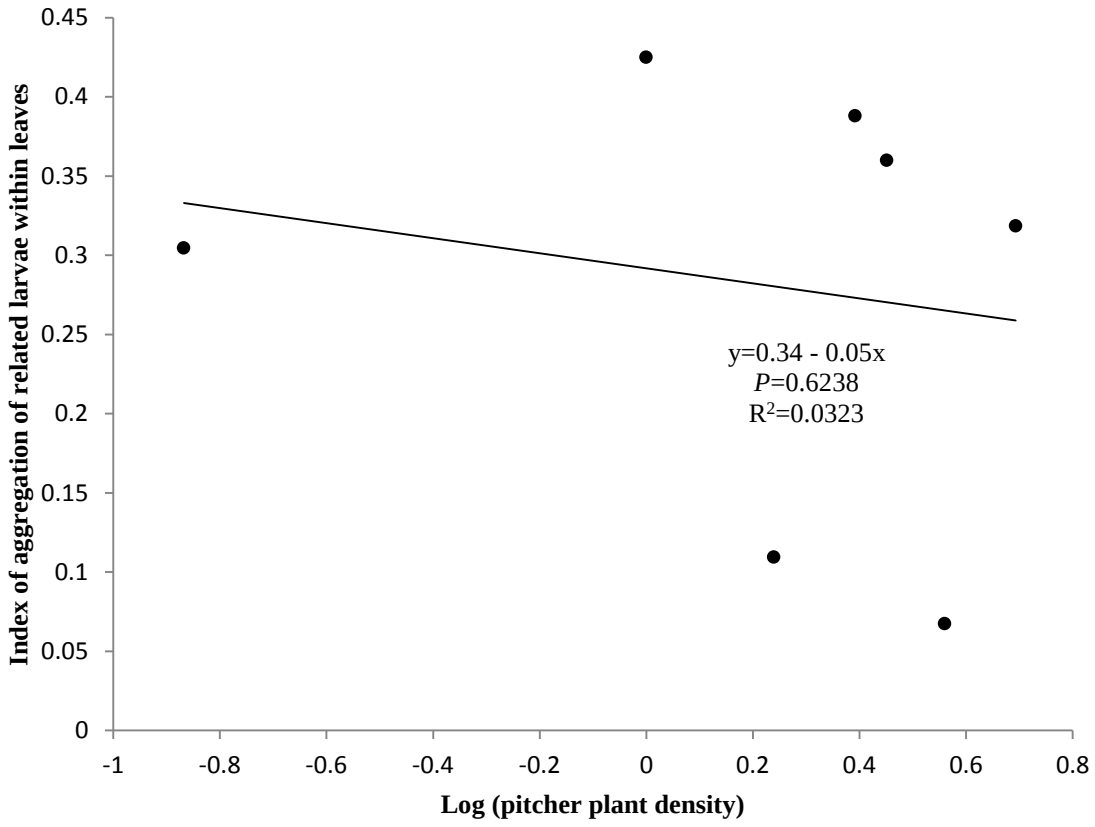

Figure 3.8 Relationship between an index of aggregation (i.e., clumping) of related larvae of the pitcher plant mosquito, *Wyeomyia smithii*, within leaves and pitcher plant density. Data are for seven peatlands from Algonquin Provincial Park (West Rose peatland is not included). The y-axis shows the mean pairwise relatedness of individual larvae sampled from different plants and clusters, subtracted from the mean pairwise relatedness of individual larvae sampled from the same leaf. The x-axis shows the natural logarithm of pitcher plant density in each peatland. Slope and intercept estimates, and P-value, are from a Generalized Linear Model with pitcher plant density and peatland area (both log transformed) as independent variables.

Chapter 4: Discussion

4.1 Broad-scale patterns of genetic structure in the pitcher plant mosquito

Contrary to my prediction, I did not observe strong genetic differentiation among populations of the pitcher plant mosquito, *W. smithii*, at broader spatial scales (among clusters within a peatland, among peatlands within a system, and between systems of peatlands). At all of these scales, global and pairwise estimates of F_{ST} and a HIERFSTAT analysis indicated weak or no differentiation. Indeed, the only instances of significant genetic differentiation I observed at the broader scales were ascribed to a single peatland, West Rose of System 2. Likewise, my prediction of patterns of isolation-by-distance (IBD) among samples from clusters and peatlands was refuted. The only situation in which I observed IBD was among peatlands from both Systems, but excluding West Rose peatland (Figure 3.2).

A number of processes could potentially explain low or no genetic differentiation among populations, in association with lack of IBD, such as I observed among clusters, peatlands, and even systems in *W. smithii*. These include similar selection pressures in different populations, very low levels of genetic drift, or high levels of gene flow. The most likely and parsimonious explanation for low genetic differentiation in *W. smithii* is high levels of gene flow.

As genome-wide markers, a large panel of AFLPs such as the one I used should, overall, reflect neutral evolutionary processes of gene flow and genetic drift rather than the process of selection. Individual AFLP loci may be linked to regions under selection and show spatial patterns of variation resulting from either divergent or homogenizing selection. However, mean patterns observed across a large panel of AFLPs are expected to reflect neutral processes that affect the entire genome. Indeed, this is the basis of using AFLPs in genome scans to detect loci under selection: individual AFLP loci that show unusually high or low differentiation among populations or groups, relative to the entire panel of a hundred or more AFLP loci, are interpreted as being linked to regions under divergent or homogenizing selection, respectively (Bonin et al. 2006). Thus, similar selection pressures in all the

sampled peatlands cannot explain the low genetic differentiation that I observed across a panel of 117 loci.

Low levels of genetic drift, and thus very high effective population sizes, can also be excluded as a main factor underlying the low genetic differentiation of *W. smithii* populations. This is based on comparison to the pitcher plant midge, *M. knabi*, another pitcher plant inquiline whose genetic structure has been studied in the same peatlands as used in my study (Rasic & Keyghobadi 2012b). In all the sampled peatlands in Algonquin Provincial Park, the population size of *M. knabi* was larger than that of *W. smithii*: when the midge and mosquito larvae were withdrawn from each pitcher, midges consistently outnumbered mosquitoes (G. Rasic, unpublished). Larger population size of *M. knabi* is very typical and has also been observed in other geographic areas; for example, in Newfoundland, Krawchuk & Taylor (2003) reported the number of *M. knabi* larvae per pitcher as being up to three times higher than the number of *W. smithii* larvae. At the same time, significant genetic differentiation was observed among *M. knabi* samples at all levels of the spatial hierarchy in the same peatlands that I studied (Rasic & Keyghobadi 2012b). If effective population sizes are not sufficiently high (i.e., levels of genetic drift are not sufficiently low) in *M. knabi* to prevent genetic differentiation, then it does not seem likely that effective population sizes would be sufficiently high to prevent differentiation in *W. smithii*, which is present in considerably smaller numbers.

Another possible explanation for my inability to detect genetic differentiation in *W. smithii* at broader spatial scales is simply that the AFLP markers I used were not sufficiently variable and therefore the data set lacked power. However, this is contradicted by my ability to observe significant differentiation among samples at the smallest spatial scale of my study, among leaves within plants (Tables 3.4-3.6). Furthermore, the levels of variability I observed at my AFLP loci, measured a proportion of loci that were polymorphic and as heterozygosity, were within the ranges typically reported in other AFLP studies. For example, in a survey of AFLP studies of Lepidoptera, reported heterozygosity values ranged from 0.031 to 0.416 (Crawford et al. 2011). The ranges of heterozygosity I observed in *W. smithii* fell within that range, being 0.116 to 0.159 and 0.063 to 0.184 when using peatlands and clusters as analysis units, respectively. Nevertheless, future studies using alternative genotyping techniques involving next-generation sequencing, such as RADSeq used by Emerson et al. (2010) in

their phylogeographic study, may provide additional insight into the fine-scale population genetic structure of *W. smithii*.

Thus, in *W. smithii,* the low genetic differentiation among clusters, peatlands and even systems of peatlands is best explained by high levels gene flow even at relatively large spatial scales (between systems). Since gene flow is mediated by dispersal, *W. smithii* is therefore seemingly easily capable of dispersing among nearby peatlands and even more distant peatlands, which counters my hypothesis that *W. smithii* has weak dispersal tendencies.

My conclusion that *W. smithii* can disperse readily among peatlands appears initially to contradict the inference of Istock & Weisburg (1987) that panmixia, or random mating, occurs only within but not among peatlands for *W. smithii.* However, Istock & Weisburg's (1987) inference of limited genetic exchange among peatlands applied mostly to a very large, continental scale. Their observation of generally low F_{ST} values at smaller scales, including a scale of up to 40 km between different peatlands, is consistent with my results. Istock & Weisburg (1987) also employed only two allozymes, which had a lower resolution than the 117 AFLP markers used in my study, and these allozymes were potentially targets of selection (Schlotterer 2004). As mentioned previously, while AFLP markers are not strictly neutral, they are genome-wide markers. Thus, a large panel of AFLPs should overall reflect neutral processes of gene flow and genetic drift. The population genetic patterns revealed by AFLPs should be less influenced by selection compared to the allele frequency patterns at one or a few enzyme loci. Indeed, my results suggest that homogeneity of enzyme allele frequencies observed among nearby peatlands by Istock & Weisburg (1987) may be partially due to high levels of gene flow, and not entirely to selection.

My results are also consistent with those of Armbruster et al. (1998) and Emerson et al. (2010), who demonstrated significant genetic structure in *W. smithii* only at very large, continental scales. Overall, my results suggest high levels of dispersal and gene flow at a spatial scale up to about 26 km in *W. smithii*.

Although I predicted that *W. smithii* should be intermediate to the other pitcher plant insect inquilines in dispersal abilities, and hence genetic structure, my results actually indicate that of all three pitcher plant insects, the mosquito shows the least genetic structure and

differentiation at larger spatial scales. The midge *M. knabi* shows significant genetic structuring at all scales of the spatial hierarchy (i.e. among leaves, plants, clusters, peatlands, and systems), whereas the flesh fly *F. fletcheri* shows little structure within peatlands, but significant differentiation among peatlands, including peatlands within the same system (i.e. within 5-7 km; Rasic & Keyghobadi 2012a). The effective mobility of adult *F. fletcheri* was previously thought to allow greater cross-peatland movement than in the smaller pitcher plant inhabitants (Dahlem & Naczi 2006). Although dispersal ability is generally thought to be correlated positively with body size in insects (Jenkins et al. 2007), the pattern is not supported here by *W. smithii*. Consequently, body size does not predict the scale of gene flow among the pitcher plant's insect inquilines.

One possible explanation for long-distance dispersal and gene flow in *W. smithii*, despite its small body size and apparently weak flight behaviour, is wind-mediated movement. Small insects and other arthropods may be passive dispersers, carried by air currents to new locations (Byrne et al. 1996). For flight-capable insects, their direction of movement over the ground can be influenced by the direction the wind is blowing (Bullock et al. 2002). Once an insect enters an air column where the air is moving more quickly than its own maximum airspeed, the insect will be carried downwind (Bullock et al. 2002). When considering *W. smithii* movement, it may be useful to consider the role of wind-assisted dispersal.

Like *W. smithii*, the carabid beetle *Notiophilus biguttatus* was previously thought to be a poor disperser present in isolated habitat patches, but Chapman et al. (2005) found that the insect can travel windborne for tens of kilometers in a single flight. Likewise, Lindsay et al. (1995) noted that the spatial distribution of *Anopheles gambiae* mosquitoes was related to the predominant wind direction at night, indicating the role of wind-mediated dispersal from breeding sites. In a phylogeographic study of *W. smithii* populations covering much of the latitudinal range of the species, Emerson et al. (2010) invoked wind-assisted dispersal as a mechanism underlying post-glacial range expansion and suggested that relationships among northern populations of *W. smithii* are consistent with such a mechanism. Perhaps wind-assisted dispersal is an important mechanism determining the genetic structure of *W. smithii* populations at smaller spatial and temporal scales as well. Future investigations into wind-assisted gene flow of *W. smithii* should incorporate the aspects of wind speeds, wind directions, and frequency of wind occurrences. More intensive sampling, at a larger spatial

scale than in my study, will likely be necessary. It will also be important to sample pairs of populations that are oriented both in the direction of, and perpendicular to, prevailing winds, in order to be able to test the hypothesis of wind-mediated gene flow.

4.2 Fine-scale patterns of genetic structure in the pitcher plant mosquito

Contrary to patterns observed at the broader spatial scales of my study, I observed strong genetic structure of *W. smithii* at the finest spatial scale, among leaves within a plant. Patterns of significant genetic differentiation among leaves (but not at larger spatial scales) most likely reflect patterns of female oviposition in the current year. Significant genetic differentiation among pitchers of a plant would arise if the larvae within each leaf are related, for example if they are siblings, and if female mosquitoes are laying their eggs in clumps within individual pitchers rather than dispersing single eggs among multiple pitchers or plants. In support of this hypothesis, the mean pairwise relatedness values between individual larvae, calculated at various scales in each peatland, indicated that relatedness of pairs of individuals was highest at the leaf compared to the broader scales (Figures 3.5 and 3.6). This pattern, and the possibility of female *W. smithii* depositing multiple eggs (i.e., offspring related as siblings or half-siblings) in the same pitcher, is not consistent with the suggestion of Heard (1994b) that females typically lay their eggs singly within pitchers. Others, however, have reported placement of multiple eggs into a single pitcher by *W. smithii* females (Bradshaw 1983). Differences between my results and the observations of Heard (1994b) may relate to differing habitat characteristics and therefore female mosquito behaviour, in the peatlands of Algonquin Provincial Park versus those of Atlantic Canada, where his study was performed. The differences may also relate to the shorter time frame encompassed in Heard's (1994b) study, which involved experimental manipulation of pitcher quality and recording of female oviposition over 3 days, compared to my study, in which larvae were collected after several possible weeks of natural female oviposition in the field.

I hypothesized that in response to low pitcher plant density, female *W. smithii* would aggregate more of their eggs into single leaves, in order to avoid moving long distances to oviposit, as observed in the midge *M. knabi* (Rasic & Keyghobadi 2012b). As predicted, I did

find a significant negative relationship between an index of aggregation, or ‘clumping’ of related larvae within leaves, and pitcher plant density (Figure 3.7). However, this relationship was driven almost entirely by a single peatland, West Rose, which had both a much higher degree of aggregation of related larvae within leaves, and a much lower plant density than all other peatlands in my study (Figure 3.8). Therefore, my analysis of the effect of pitcher plant density on oviposition patterns of female *W. smithii* is inconclusive. Further study, using more peatlands with low plant density, is required to resolve the relationship.

Interestingly, West Rose peatland stood out in my study for a number of reasons. In addition to driving the relationship between relatedness of larvae within leaves and pitcher plant density, *W. smithii* samples from West Rose peatland also had higher genetic diversity, lower overall relatedness of individuals, and higher genetic differentiation from other peatlands. For *M. knabi*, samples from West Rose peatland were also highly differentiated from all other peatlands, even nearby ones (Rasic & Keyghobadi 2012b). West Rose peatland differs from all other study peatlands in that it is essentially an island mat of sphagnum surrounded by water, in addition to having very low plant density compared to the other peatlands. These factors may be influencing genetic structure of *W. smithii* as well as *M. knabi*. Further sampling of peatlands with similarly low pitcher plant densities and/or isolation by water would be helpful in determining whether these are indeed the factors contributing to the unusual genetic make-up of insect populations in West Rose peatland.

Summary

I elucidated the population genetic structure of the pitcher plant mosquito *W. smithii* using 117 AFLP markers derived from four selective primer combinations. Unexpectedly, there was very little structure and very low genetic differentiation among *W. smithii* samples at all scales, save for among leaves within plants. Although *W. smithii* is traditionally thought to be a poor disperser, my results indicate that there are likely high levels of gene flow among peatlands even up to 26 km apart. I inferred higher levels of movement and gene flow at larger scales in *W. smithii* than has been observed in the other pitcher plant insect inquilines, the midge *M. knabi* and the flesh fly *F. fletcheri*. Among these insects, it appears that body size is not a predictor of dispersal ability. High gene flow among peatlands could be a consequence of wind-assisted dispersal of *W. smithii*, a hypothesis that warrants further study.

In association with significant genetic differentiation among samples of *W. smithii* collected from different leaves of the same plant, the average degree of relatedness of pairs of individuals was greatest at the leaf scale, compared to the broader scales. These results suggest that female mosquitoes are laying their eggs in clumps within individual leaves instead of dispersing eggs singly among multiple pitchers. I observed a significant negative relationship between pitcher plant density and the degree of clumping of eggs within leaves (i.e., the degree relatedness of larvae within leaves relative to broader scales), but this was driven primarily by West Rose peatland, which had both a much lower pitcher plant density than all other peatlands in my study and showed much higher relatedness of larvae at the leaf scale compared to broader scales. West Rose peatland was also unusual in that *W. smithii* samples from that peatland displayed high genetic diversity, low relatedness of individuals, and high differentiation from other peatlands (F_{ST} values), which perhaps could be explained by the peatland's lower pitcher plant density and isolation by water. Therefore, the relationship between plant density and egg clumping is inconclusive; future studies should sample mosquitoes from peatlands with a similarly low plant density as West Rose peatland, in order to resolve this unclear relationship.

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Related Work Experience

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